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# Localized Tetrazolium Reduction in Relation to $N_2$ Fixation, $CO_2$ Fixation, and $H_2$ Uptake in Aquatic Filamentous Cyanobacteria

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The aquatic filamentous cyanobacteria *Anabaena oscillarioides* and *Trichodesmium* sp. reveal specific cellular regions of tetrazolium salt reduction. The effects of localized reduction of five tetrazolium salts on $N_2$ fixation (acetylene reduction), $^{14}CO_2$ fixation, and $^{3}H_2$ utilization were examined. During short-term (within 30 min) exposures in *A. oscillarioides*, salt reduction in heterocysts occurred simultaneously with inhibition of acetylene reduction. Conversely, when salts failed to either penetrate or be reduced in heterocysts, no inhibition of acetylene reduction occurred. When salts were rapidly reduced in vegetative cells, $^{14}CO_2$ fixation and $^{3}H_2$ utilization rates decreased, whereas salts exclusively reduced in heterocysts were not linked to blockage of these processes. In the nonheterocystous genus *Trichodesmium*, the deposition of reduced 2,3,5-triphenyl-2-tetrazolium chloride (TTC) in the internal cores of trichomes occurs simultaneously with a lowering of acetylene reduction rates. Since TTC deposition in heterocysts of *A. oscillarioides* occurs contemporaneously with inhibition of acetylene reduction, we conclude that the cellular reduction of this salt is of use in locating potential $N_2$-fixing sites in cyanobacteria. The possible applications and problems associated with interpreting localized reduction of tetrazolium salts in cyanobacteria are presented.

Tetrazolium salts are heterocyclic organic compounds capable of forming insoluble colored crystals, termed formazans, on reduction. Formazan deposition is a useful indicator of reduced conditions in microenvironments or microzones since deposits indicate specific redox potentials and can be readily detected with the light microscope. Formazan crystal sizes range from one to several micrometers in diameter, making it possible to observe deposition in cellular organelles and near membrane structures in eucaryotic cells, and in specific reduced subcellular regions in procaryotic cells.

The use of tetrazolium salts in organic chemistry was initiated in 1894 when von Pechman and Runge (26) synthesized 2,3,5-triphenyl-2-tetrazolium chloride (TTC). It was not until the early 1940s that the usefulness of these salts was realized in biological studies, when Kuhn and Jerchel (13) discovered the ability of several tetrazolium salts to produce a mutagenic effect on green algae. Further work (13) revealed the ability of a range of microorganisms to biologically reduce 2,3-diphenyl 5-hexyltetrazolium chloride to formazan crystals. Current work has linked reduction to specific biochemical electron transfer reactions and to the cellular locations of such reactions (1, 2, 15).

Tetrazolium salts have only recently received use in studies of the physiological ecology of algae and bacteria. Among these are: the study by Stewart et al. (24) on TTC inhibition of nitrogenase activity (NA) but not $CO_2$ fixation activity in filamentous heterocystous cyanobacteria; the demonstration by Fay and Kulasooriya (10) that TTC shows specific formazan deposition in the highly reduced heterocyst of the nitrogen-fixing cyanobacterium *Anabaena* sp. with simultaneous inhibition of $N_2$ fixation (acetylene reduction); and the use by Zimmerman et al. (28) of 2-(*p*-iodophenyl) 3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to distinguish respiring from nonrespiring aquatic bacteria.

We used five tetrazolium salts with a range of redox potentials as indicators of specific sites of biological reduction in the freshwater filamentous bloom-forming cyanobacterium *Anabaena oscillarioides* and the marine filamentous cyanobacterium *Trichodesmium* sp. Visual observations of tetrazolium salt reduction (formazan deposition) were coupled to determinations of NA, $CO_2$ fixation, and $H_2$ utilization. Our work is an evaluation of the usefulness of a range of salts in visually locating metabolically active cellular regions and their potential for supporting the measured activities.

## MATERIALS AND METHODS

*A. oscillarioides* was isolated from the Waikato River, New Zealand, and was donated as axenic filaments by K. Lam, Ministry of Works, Hamilton, New Zealand (Ph.D. thesis, University of Auckland, New Zealand, 1978). Cultures were grown in Chu-10 (nitrogen free) medium (7) on a shaker table under a regimen of 14 h of light followed by 8 h of darkness. Illumination was provided by cool white fluorescent lamps at 2,000 lx. Freshly sampled *Trichodesmium* sp. was obtained from Bogue Sound near the Atlantic coast of North Carolina. *Trichodesmium* sp., was employed in a single experiment, conducted during a $N_2$ fixation bloom on 24 August 1980.

The following tetrazolium salts were all obtained from Sigma Chemical Co., St. Louis, Mo. (pfs grade) and are listed in order of increasing redox potential: INT, −0.05 V; 3,3-(3,3-dimethoxy-4,4-biphenylylene)-bis-2,5,-*p*-nitrophenyl-SH-tetrazolium chloride) (TNBT), −0.02 V; 3,3-(3,3-dimethoxy-4,4-biphenylylene)-bis-(2-*p*-nitrophenyl-5- phenyl-2H-tetrazolium chloride) (NBT), −0.01 V; 3α-naphthyl-2.5-diphenyl-2H-tetrazolium chloride (TV), +0.29 V; TTC, +0.40 V. TTC, TV, and INT were added at a final concentration of 0.01% (wt/vol), whereas NBT and TNBT were added at 0.005% (wt/vol). Reduced concentrations were employed for the latter two salts after they proved to be toxic at 0.01%. Care was taken to minimize salt exposure to light; all salt solutions were stored refrigerated in foil-wrapped reagent bottles. As a check on nonbiological photo or chemical reduction of tetrazolium salts, 3% (wt/vol) buffered (pH 7.5) Formalin-killed *A. oscillarioides* cultures received salt additions identical to concentrations used in live cultures.

The effects of tetrazolium salts on $N_2$ and $CO_2$ fixation as well as $H_2$ uptake were most extensively studied in *A. oscillarioides*. Incubations were done in either 30- or 60-ml clear-glass serum bottles placed on an orbital shaker under 2,000-lx cool white fluorescent light. To terminate tetrazolium salt incubations, a final concentration of 3% buffered (pH 7.5) glutaraldehyde was introduced into serum bottles. Samples could then be stored indefinitely for observations of formazan crystal deposition with a Zeiss phase-contrast microscope at ×1,000.

Triplicate NA, $^{14}CO_2$ fixation, and $^3H_2$ utilization-exchange assays were conducted in conjunction with each tetrazolium salt addition. NA was assayed by the acetylene reduction method as originally described by Stewart et al. (23) and modified by Paerl and Kellar (16). An AGC 311 flame ionization gas chromatograph (Carle, Anaheim, Calif.) with a 2-m Poropak T (Waters Associates, Milford, Mass.) column heated to 80°C was used for detecting ethylene production. Molar ratios of acetylene reduced versus $N_2$ fixed were determined by performing parallel $^{15}N_2$ fixation assays on several occasions. Incorporation of $^{15}N_2$ (added at 90% atomic excess) by *A. oscillarioides* was monitored by the use of an emission spectrophotometer capable of discriminating spectral differences between $^{14}N_2$, $^{15}N$ $^{14}N$, and $^{15}N_2$ after high-voltage atomic excitation of samples in a Jasco N1A-1 emission spectrophotometer (12).

Photosynthetic carbon fixation was measured by detecting the incorporation of $^{14}C$-labeled sodium bicarbonate (Amersham Corp., Arlington Heights, Ill.) added to stoppered serum bottles containing *A. oscillarioides*. Radioisotope was added at 2 μCi/50 ml of culture. After incubation intervals of up to 2 h, samples of culture were withdrawn by syringe, filtered at 200-Torr vacuum through 25-mm Whatman GFC glass fiber filters, and then rinsed with 5 ml of unlabeled Chu-10 medium. Filters were then air dried, fumed for 10 min in an HCl atmosphere to remove precipitated $^{14}C$, and then placed under a ventilated hood for 1 h to remove traces of HCl. Filters were placed in scintillation cocktail (Fisher Scientific Co., Raleigh, N.C.). All samples were counted in an LS 7000 liquid scintillation system (Beckman, Fullerton, Calif.) at efficiencies ranging from 90 to 95%.

A tritium exchange assay as initially described by Lim (14) and Chan et al. (6) was used to measure $H_2$ utilization by *A. oscillarioides*. The method monitors the exchange reaction between tritium gas and water, which is mediated by uptake hydrogenases. Since nitrogenase is known to produce $H_2$ (19, 25), the utilization of $H_2$ may be related in time and cellular locality to $H_2$ production. We injected 250 μl of $^3H_2$ (New England Nuclear Corp., Boston, Mass.) (specific activity, 3 mCi/ml) into 30-ml sealed serum bottles, each containing 25 ml of *A. oscillarioides* culture. Bottles were placed on an orbital shaker, and the aqueous phase was periodically sampled for $^3H_2O$ accumulation. Subsamples (0.5 ml) were withdrawn by syringe at prescribed intervals and dispensed in liquid scintillation vials. Excess $^3H_2$ was allowed to escape from the sample by leaving the vials uncapped for 10 min under a vented fume hood. The remaining $^3H_2O$ was then counted in a dioxane-based water-miscible liquid scintillation cocktail (H. W. Paerl, Ph.D. thesis, University of California, Davis, 1973) at 42% efficiency.

## RESULTS AND DISCUSSION

**Formazan deposition in *A. oscillarioides*.** Visual observations were made every 10 min after tetrazolium salt additions. Formazan deposition patterns for individual salts were as follows.

**TNBT.** Fine TNBT formazan deposits, appearing purplish-red, were rapidly formed in vegetative cells of *A. oscillarioides*. Within the first 10 min, vegetative cells showed general darkening, particularly near the cellular junctions and just inside the cell wall (Fig. 1). Vegetative cells bordering the heterocysts showed the most extensive deposition. During the first 30 min, deposition grew more intense in all of the above-mentioned regions. After 30 min, *A. oscillarioides* was adversely affected by TNBT, as exemplified by filament breakage. TNBT failed to show formazan deposition in heterocysts throughout the duration of observations.

**NBT.** Depositional patterns for NBT were similar to those for TNBT, although the intensity of deposition often varied between these salts. As with TNBT, vegetative cells showed rapid darkening, particularly in cells forming the termini of filaments and in cells bordering het-

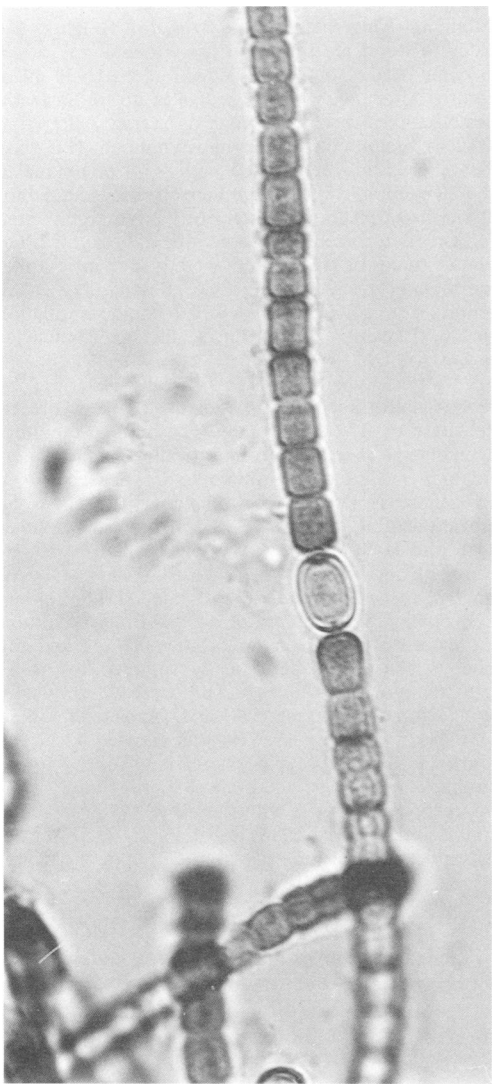

FIG. 1. Darkening of vegetative cells of *A. oscillarioides* 10 min after exposure to TNBT. Note the particularly dark vegetative cells bordering the heterocyst (transparent thick-walled cell located midway in this filament). Depositional patterns of NBT formazan crystals were quite similar to those observed for TNBT. The length of each vegetative cell is approximately 4 μm.

erocysts. Very small granules of less than 0.2 μm in diameter and having a purplish-blue color were deposited. Depositional patterns were well established after 20 min of exposure. After 40 min, filament breakage was commonly observed. Indications were that prolonged (more than 30 min) exposures to 0.005 to 0.01% NBT were toxic and eventually destructive to *A. oscillarioides*. No NBT deposition was observed in heterocysts at any time after salt addition.

**INT.** Granular brownish-red deposits were observed throughout vegetative cells within the first 10 min of INT formazan deposition. Deposits appeared uniform in intensity among all vegetative cells, including those bordering heterocysts (Fig. 2). Formazan grain size was 0.2 to 0.5 μm in diameter. Throughout the first 60 min, grain density steadily increased until filaments were a solid brown due to heavy deposition. Heterocysts failed to show formazan deposition during the initial 30 min, after which small deposits began to occur near the polar regions of heterocysts. Structural damage, such as filament breakage and lysis, was not observed within a 2-h exposure period.

**TV.** Between 10 and 20 min after the addi-

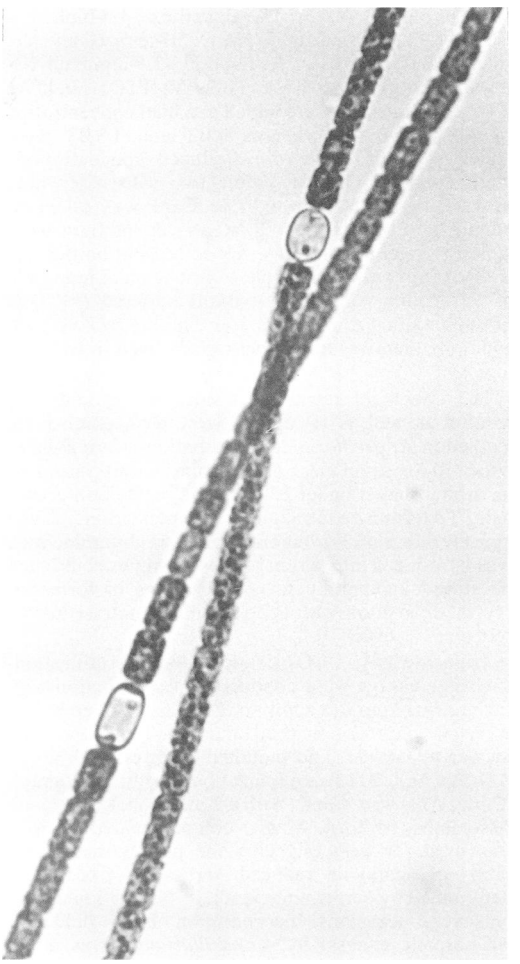

FIG. 2. Granular INT formazan deposition throughout the vegetative cells of *A. oscillarioides*. Heterocysts remained free of deposits, the only noticeable darkening being the polar plug regions adjoining vegetative cells. This photomicrograph was taken 12 min after exposure to INT.

FIG. 3. Deposition of needle-like TV formazan crystals throughout both the heterocyst and vegetative cells of an *A. oscillarioides* filament. This filament was photographed 25 min after initial exposure to TV.

tions, large orange-red needle-like TV formazan crystals appeared in vegetative cells. After 20 min, such crystals were also seen in heterocysts (Fig. 3). After 30 min, large formazan crystals were commonly observed throughout filaments. Crystal size was variable, ranging from 1 to 5 μm. Structural damage was not observed within a 2-h exposure period.

**TTC.** During the first 10 min of exposure, slight TTC formazan crystal deposits were observed only in heterocysts. Deposits initially appeared as red needle-like crystals concentrated in the polar regions of heterocysts (Fig. 4). Deposits were more pronounced in heterocysts after 20 min, often filling the heterocysts with large, needle-like crystals of from 1 to 5 μm in length. After 20 min, slight TTC formazan deposition was also observed in vegetative cells. After 60 min, heterocysts were filled with large crystals, whereas vegetative cells showed scattered but not heavy crystal deposition. Filaments remained intact throughout a 2-h incubation period.

**Formalin-killed samples.** After the addition of 3% buffered Formalin, none of the tetrazolium salts revealed subsequent cellular formazan de-

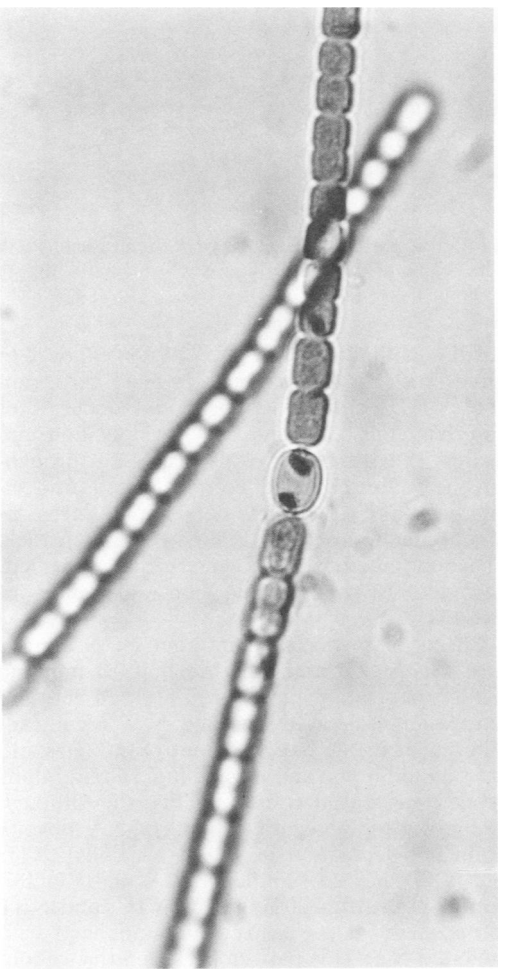

FIG. 4. Initial deposition of TTC formazan crystals in the polar regions of *A. oscillarioides* heterocysts. Photograph was taken 10 min after initial exposure to TTC. Eventually, entire heterocysts became filled with dark-red TTC formazan crystals, whereas vegetative cells began to show small amounts of crystal deposition after 20 min of exposure.

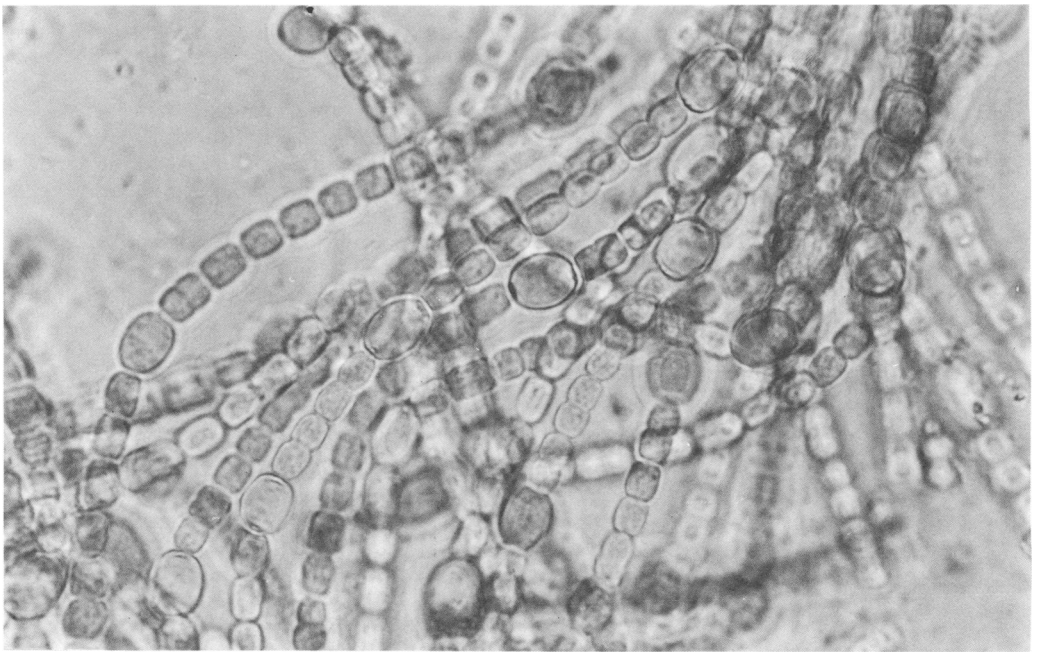

FIG. 5. Formalin-killed *A. oscillarioides* filaments after a 60-min incubation with a mixture of five tetrazolium salts. None of the salts added were reduced within this period, leaving the filaments formazan-free.

position within 60 min (Fig. 5). When exposed to three times the light intensities used here, random TTC formazan deposits did occur after approximately 30 min. Such deposition appeared as large red crystals dispersed throughout the culture, most often not located intracellularly. We regarded such deposition patterns as photoreduction of TTC at high light intensities. These potential artifacts were easily remedied, however, by maintaining light intensities of 2,000 lx.

**Effects of formazan deposition on NA in *A. oscillarioides*.** Throughout the first 10 min, no significant relationships were observed between tetrazolium salt additions and NA. Significant differences in NA between control (no tetrazolium salt additions) and tetrazolium salt additions became evident after 10 min (Fig. 6). Although INT revealed no negative effects on NA (despite its heavy deposition in vegetative cells), TTC and NBT showed significant ($P < 0.01$) inhibition of NA. After 20 min, TV, TTC, and NBT formazan deposits occurred simultaneously with inhibited NA. This pattern persisted throughout the 30-min experimental period. It appeared that NBT was toxic to *A. oscillarioides* after 10 min, completely arresting $O_2$ evolution and carbon fixation and inducing fragmentation of filaments. Hence, it was not surprising to note a cessation of NA after 10 min as well. The inhibitory effects of TTC and TV were not due to toxicity, however, since other metabolic functions (such as $O_2$ evolution) did not cease. There was a strong correlation ($r^2 = 0.92$; degree of freedom = 24) between the appearance of TTC and TV formazan crystals in heterocysts and the inhibition of NA in all experiments. Stewart et al. (24) and Fay and Kulasooriya (10) noted a similar relationship between TTC deposition and the inhibition of NA in *Anabaena cylindrica*. The relationship between TTC deposition and NA inhibition was more closely examined over a set of time intervals. As signs of formazan deposition appeared initially in the polar regions of the heterocyst, NA inhibition proceeded (Table 1). The inhibition appeared nearly complete after 50 min, at which time virtually all heterocysts were filled with TTC formazan crystals (Table 1).

A contrasting situation resulted with INT formazan deposition. As INT deposition exclusively progressed in vegetative cells for up to 20 min, no inhibition of nitrogenase activity was observed. Between 20 and 30 min, traces of INT formazan crystals began to appear in heterocysts. During this interval, slight inhibition of NA became evident.

The monitoring of $^{15}N_2$ uptake in parallel with acetylene reduction assays yielded molar ratios (of acetylene reduced to fixed $^{15}N_2$) for *A. oscillarioides* ranging from 2.71 to 4.15, approximating a molar conversion based on a theoretical ratio of 3:1 (11, 21). The average molar ratio

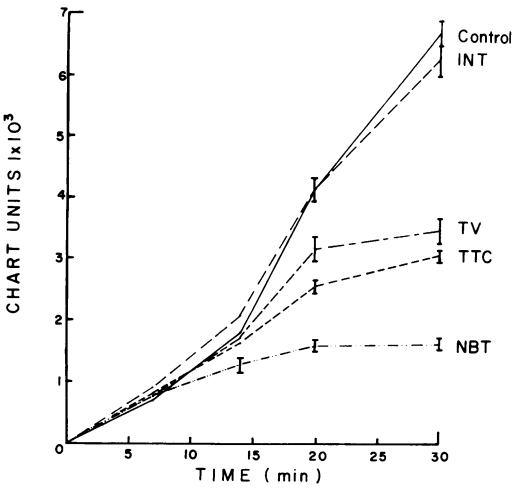


FIG. 6. Effects of various tetrazolium salts on acetylene reduction in *A. oscillarioides*. Acetylene reduction results are given in recorder chart units of ethylene produced as measured by gas chromatography. Vertical error bars represent the extreme differences between triplicate samples. At the intervals between 0 and 10 min where error bars were omitted, no significant differences between control, INT, TV, and TTC were found.

derived from all values was 3.31, slightly in excess of the theoretical ratio. These findings are in reasonable agreement with a ratio of 4:1 found by Peterson and Burris (18). The fact that the present and previous studies lead to ratios in excess of 3:1 would indicate that $H_2$ production via nitrogenase activity is actively occurring in *Anabaena* populations (Paerl, manuscript in preparation).

In general, the effects of TTC and TV formazan deposition in heterocysts on NA substantiate the commonly held belief that NA is largely confined to the heterocysts of *Anabaena* spp. during aerobic growth (8, 11, 21, 25). Because of their relatively low molecular weights, TTC (334.8) and TV (384.9) can most readily diffuse into the heterocysts. These salts also require the highest redox potential (most difficult to reduce) for reduction and formazan deposition. Hence, they are most suitable in competing for electrons with a process requiring highly reduced conditions, such as NA. Although these tetrazolium salts are not known to be specific inhibitors of NA, the strong correlation between their deposition in heterocysts and the cessation of NA suggest that the heterocysts are the sites of competition for reducing power between tetrazolium reduction and nitrogen fixation.

We also examined the effects of TTC formazan deposition on acetylene reduction and $^{14}CO_2$ fixation during a bloom of the nonheterocystous marine cyanobacterium *Trichodesmium* sp. Carpenter and Price (5) reported detectable $N_2$ fixation rates when this genus was present in a colonial form composed of bundles of individual filaments. When the bundles were disrupted or when individual filaments were present, $N_2$ fixation abruptly ceased. Carpenter and Price (5) suggested that $N_2$ fixation was promoted by bundle formation because such a structural conformation allowed the inner core of the bundles to become reduced. This was accomplished through reduced photosynthetic $O_2$ evolution in the core when compared with $O_2$ evolution in peripheral filaments. If this explanation is correct, one would expect to find more extensive

TABLE 1. Progressive inhibition of NA (acetylene reduction) by TTC in *A. oscillarioides*

| Time after TTC addition (min) | Visual observations | % Inhibition[a] |
|---|---|---|
| 10 | No signs of formazan deposition anywhere in *Anabaena* filaments | 2 (NS) |
| 20 | Formazan deposition in polar areas of heterocysts; approximately 30% of heterocysts showed deposition | 21 (<0.01) |
| 30 | Virtually all heterocysts showed formazan deposition; large crystals developing; no deposition in vegetative cells | 53 (<0.001) |
| 40 | Heterocysts showed heavy formazan deposits; approximately 10% of vegetative cells now showed deposits | 72 (<0.001) |
| 50 | Heterocysts filled with formazan deposits; approximately 30% of vegetative cells showed deposits | 85 (<0.001) |
| 60 | Formazan deposits throughout the filaments, but deposition still heaviest in heterocysts | 89 (<0.001) |

[a] Compared with control samples. The degrees of difference are shown within parentheses. NS, Not significant.

TABLE 2. Progressive inhibition of NA (acetylene reduction) and $^{14}CO_2$ fixation by TTC in *Trichodesmium* sp.

| Time after TTC addition (min) | Visual observations | % Inhibition[a] of acetylene reduction | % Inhibition[a] of $CO_2$ fixation |
|---|---|---|---|
| 10 | No formazan deposition observed | 1 (NS) | 0 (NS) |
| 20 | Small formazan crystals becoming apparent in the inner core of bundles | 31 (<0.001) | 2 (NS) |
| 30 | Extensive formazan deposition observed in inner core; no deposition evident in peripheral regions | 72 (<0.001) | 5 (<0.05) |
| 40 | Virtually all cells in inner core showing heavy formazan deposits; peripheral cells beginning to show slight deposits | 91 (<0.001) | 27 (<0.001) |
| 50 | Core cells filled with formazan deposits; peripheral cells now showing heavy deposits | 93 (<0.001) | 59 (<0.001) |
| 60 | Core and peripheral cells filled with formazan deposits | 95 (<0.001) | 83 (<0.001) |

[a] See Table 1, footnote *a*.

deposition of TTC formazan crystals in the core as opposed to peripheral areas of bundles. After the addition of 0.01% (wt/vol) TTC as prescribed for *A. oscillarioides*, we observed TTC formazan deposition. Indeed, TTC deposition was confined to the core areas throughout the first 30 min of exposure. Furthermore, when deposition initially appeared in the core areas, acetylene reduction was strongly inhibited (Table 2). Subsequent deposition in peripheral areas did not appear to be related to acetylene reduction rates. The converse was true when $^{14}CO_2$ fixation was monitored. Initial formazan deposition in core areas had virtually no inhibitory effect on $^{14}CO_2$ fixation, whereas inhibition of $^{14}CO_2$ fixation commenced as soon as TTC deposition could be seen in peripheral areas (Table 2).

**Formazan deposition and photosynthetic $CO_2$ fixation in *A. oscillarioides*.** Inhibition of $CO_2$ fixation in *A. oscillarioides* was temporally related to formazan deposition in vegetative cells. This was observed after TV, NBT, TNBT, and INT additions (Fig. 7). These salts were rapidly (within 10 min) reduced in vegetative cells, whereas TTC failed to show deposition in vegetative cells until 30 min after addition. With the onset of TTC formazan deposition in vegetative cells after 30 min, significant ($P < 0.01$) inhibition of $CO_2$ fixation occurred. When TTC deposits were present solely in heterocysts, $CO_2$ fixation was unaffected. These results provide visual evidence that the two fixation processes are spatially separated in *A. oscillarioides*, confirming earlier studies based on biochemical and physiological techniques or physical separation

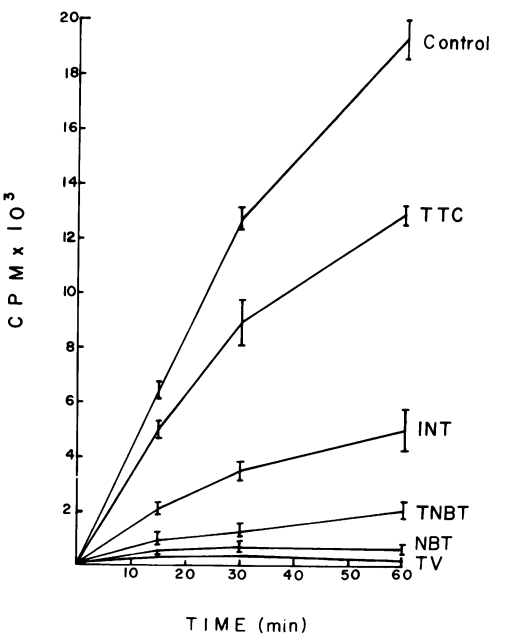


FIG. 7. Relationships between $^{14}CO_2$ fixation, reported as $^{14}C$ assimilation counts per minute, and the addition of various tetrazolium salts in *A. oscillarioides*. Vertical error bars represent the extreme values of triplicate samples. Both INT and NBT applications significantly ($P < 0.001$) and immediately inhibited $^{14}CO_2$ assimilation when compared with control conditions. The inhibitory effects of TTC were not significant, however, until after 30 min of exposure, at which time noticeable formazan deposition occurred in vegetative cells.

of filaments into vegetative cells and heterocysts (8, 22).

**Effects of tetrazolium salts on $^3H_2$ uptake in *A. oscillarioides*.** The localization of uptake hydrogenases has been under study in $N_2$-fixing cyanobacteria, since these enzymes may be linked to the source of $H_2$ production, namely nitrogenase. Previous work has suggested that the heterocysts of *Anabaena* spp. are particularly active $H_2$ uptake sites (19, 20, 25). Peshek (17) found functionally distinct uptake hydrogenases in cyanobacteria, both in heterocystous and nonheterocystous genera, whereas vegetative cells of *Anabaena* spp. are also thought to possess uptake hydrogenases (20). Furthermore, Bothe et al. (3, 4) and Eisbrenner and Bothe (9) have shown that $H_2$ uptake proceeds under aerobic conditions in *Anabaena* spp. in a Knallgas-type reaction, wherein $O_2$ is consumed and ATP is generated from the oxidation of $H_2$. The utilization of $H_2$ shows a similar $O_2$ dependency in *A. oscillarioides*, which is discussed in a separate study (Paerl, Ustach, and Bland, manuscript in preparation). The end product of the Knallgas reaction is $H_2O$. Given this pathway of $H_2$ utilization in *Anabaena* spp., we investigated the use of tetrazolium salts in locating potential sites of $H_2$ uptake activity.

A strong relationship was observed between INT and NBT formazan deposition and inhibition of $^3H_2$ uptake (Fig. 8). Both NBT and INT formazan deposits were exclusively located in the vegetative cells. Conversely, TTC formazan, which was largely confined to heterocysts during the initial hour of exposure, consistently failed to inhibit $^3H_2$ uptake. This finding was surprising since we assumed that a portion of the $H_2$ utilized in *A. oscillarioides* might have come from nitrogenase. Repeatedly, however, TTC and TV formazan deposition, which also adversely affected NA, had no negative effect on $H_2$ uptake. Both TTC and TV are electron acceptors. Hence, a priori, there is little reason to suspect that they would compete with $H_2$ utilization, since $H_2$ serves as a potential electron donor. However, if cellular $H_2$ production is dominated by nitrogenase, which is a potent electron acceptor, any inhibition of NA would have a marked effect on lowering $H_2$ availability. Results obtained here indicate that although NA inhibition is closely coupled to TTC and TV deposition, parallel cessation of $H_2$ utilization (due to a lack of $H_2$ formation by nitrogenase) was not observed. Our findings suggest that $H_2$ uptake may not be solely confined to the heterocysts. A close relationship between $H_2$ formation by nitrogenase and $H_2$ uptake by hydrogenases located in the heterocysts could not be confirmed by these experiments. Results lend support to the conclusions of Bothe et al. (3, 4)

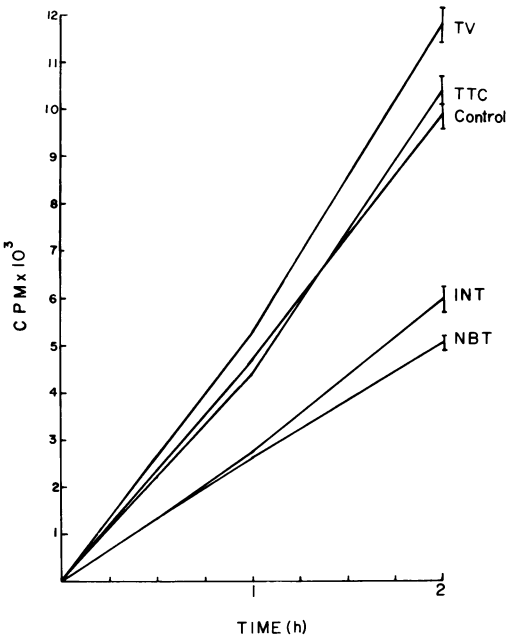


FIG. 8. Effects of tetrazolium salt additions on $^3H_2$ uptake in *A. oscillarioides*. Both INT and NBT proved to be significant inhibitors of this process when compared with control conditions ($P < 0.001$ at both 1-h and 2-h samplings). Conversely, TV and TTC failed to inhibit $^3H_2$ uptake at all times. Vertical bars, plotted at the 2-h sampling interval, illustrate the extreme values among triplicate samples run for each treatment.

as well as our own results (Paerl, Ustach, and Bland, manuscript in preparation) that an oxygen-dependent Knallgas reaction coupled to an oxygen-tolerant uptake hydrogenase may be an important means of consuming $H_2$ in *Anabaena* spp. Such a system could be functioning in subcellular microzones in vegetative cells, where photosynthetically generated oxygen supplies are plentiful but must be compatible with $O_2$ requirements for uptake hydrogenases. If the Knallgas reaction is poised at removing oxygen, thereby detoxifying *Anabaena* spp. from high intracellular oxygen concentrations (3), the vegetative cells would be an attractive site for such a reaction to take place. Clearly, more work is needed to clarify these observations. The present application of tetrazolium salts is limited to a test for possible interactions with $H_2$ metabolism.

Short-term exposures, lasting less than 30 min, appeared to be most useful for localizing metabolic functions in *A. oscillarioides* and *Trichodesmium* sp. Longer exposures often lead to complications which can be caused by several factors. (i) Toxicity and structural damage, particularly noticeable with TNBT and NBT additions, can cause cessation of all metabolic func-

tions, making useful interpretations impossible. (ii) Since photosynthesis and $N_2$ fixation can require a common source of reducing power (light-driven photolysis of water, for example), inhibition of one process can, over time, affect the performance of the other. (iii) Nitrogen fixation relies on $CO_2$ fixation as a source of carbon skeletons and reduced organic compounds. When appreciable carbon transfer from vegetative cells to heterocysts occurs, location of these processes based on formazan deposition will become exceedingly difficult. (iv) The redox potentials of specific tetrazolium salts are often favorable for simultaneously competing with several biochemical reactions in terms of electron transfer. Multiple sites of interaction with electron transport systems can occur. Specific sites of interaction do exist for certain tetrazolium salts. A classic example is the mitochondrial succinoxidase system, where specific inhibitors are used to locate the steps in this system susceptible to tetrazolium salt interaction (15). Such examples are an exception rather than a rule, however. (v) Only high-purity tetrazolium salts must be used, since impurities, particularly if they are other tetrazolium salts, can lead to incorrect interpretations of inhibitory effects.

Despite these drawbacks, tetrazolium salts can be shown to be highly useful as a rapid, simple, and readily detectable means of locating areas of major metabolic functions in microorganisms. They are particularly useful among filamentous or colonial microorganisms, wherein specific functions are often confined to distinct localities. We are currently expanding the applications of tetrazolium salts, employing formazan deposition on sediment and detritus particles as a rapid indicator of microzones harboring general microbial activity and microbially mediated nutrient-transforming reactions.

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